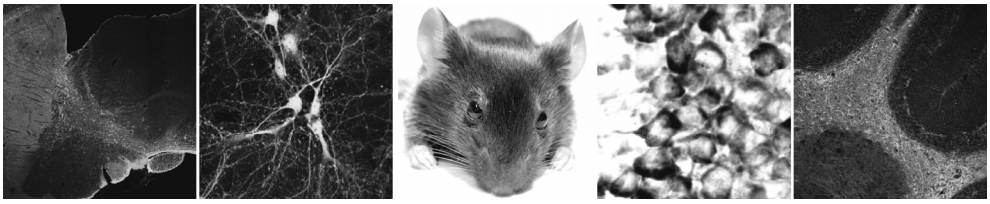
Chapter 1



Introduction and aim of this thesis

Contents:

1. cAMP signaling in learning and memory

In the molecular mechanisms that underlie the formation of all memories from simple adaptive responses to complex declarative memories, nerve cells and their interacting functional networks are subject to a large variety of signaling molecules including neurotransmitters, hormones, cytokines and matrix molecules. All these messengers can exert their influence to the same nerve cell. At the same time each nerve cell according to the model of Hebb can participate in different memory traces and respond to a variety of temporal and spatial dynamics. An obvious question then coming up is how a single individual neuron can handle the myriads of stimuli necessary to build memories during life, or in other words: how do nerve cells organize the where and when in their extensive cellular domain?

So far, a large number of signalling proteins have been shown to be involved in learning and memory processes. One of the most important molecules involved is cyclic adenosine monophosphate (cAMP, cyclic AMP or 3'-5'-cyclic adenosine monophosphate), a second messenger ubiquitously expressed and shown to be involved in many other biological processes besides learning and memory. This is e.g. evidenced by the fact that there are more than 90.000 scientific publications concerning cAMP since its discovery as a diffusible intracellular second messenger produced in response to hormone action (Sutherland and Rall, 1958). In the brain, activation of cAMP signaling occurs after stimulation of adenylyl cyclases by stimulatory G-proteins after binding of an extracellular ligand (e.g. adrenaline) to a GPCR and by Ca^{2+} through the Ca^{2+}-binding protein calmodulin (Wang and Storm, 2003).
Initially, the importance of cAMP signaling in learning and memory was demonstrated in genetic and pharmacological studies in invertebrate models for associative learning such as Aplysia (Byrne and Kandel, 1996), Drosophila (Waddell et al., 2000), and in honeybees (Apis mellifera) (Hammer and Menzel, 1995). Soon after, evidence for the role of cAMP in learning and memory in mammals was published. Genetic manipulations of several components of the cAMP pathway, such as augmentation of adenylyl cyclase activity (Pineda et al., 2004) or altering the expression of G-proteins (Bourtchouladze et al., 2006),

led to severe memory impairments in mice. Although cAMP was acknowledged as crucial molecule in learning and memory, the precise role of cAMP in these processes remained unclear due to contradictory results from other pharmacological and genetic studies. While the elevation of cAMP levels in the hippocampus by forskolin, an activator of adenylyl cyclase, improved memory for passive avoidance tasks in rats (Bernabeu et al., 1997), pharmacological activation of the cAMP pathway in the prefrontal cortex was shown to impair working memory performance (Taylor et al., 1999). Very recently, it was shown that transgenic mice in which cAMP levels are spatially and temporally elevated in forebrain regions, exhibit enhanced memory consolidation and retrieval for contextual fear conditioning (Isiegas et al., 2008). Altogether, these results suggest that cAMP signals are mediated by different intracellular mechanisms and their actions dependent on the brain region involved. Thus cAMP appears to play a complex role in distinct cognitive processes in different brain regions.

In this thesis we will investigate the role of two, recently discovered, cAMP signaling avenues, A-kinase anchoring proteins (AKAPs) and exchange protein activated by cAMP (Epac), in memory processes.

2. Cyclic AMP dependent protein kinase

One of the main targets of cAMP is cAMP-dependent protein kinase (PKA). Mammalian PKA includes four regulatory (RIα, RIβ, RIIα, RIIβ) and three catalytic (Cα, Cβ, Cγ) subunits, each encoded by a separate gene (McKnight et al., 1988; Doskeland et al., 1993). Two major types of mammalian PKA, type I (PKA I, with RIα and RIβ dimers) and type II (PKA II, with RIIα and RIIβ dimers), were initially described by their pattern of elution from diethylaminoethylcellulose (DEAE) cellulose columns (Tasken et al., 1993, Francis and Corbin, 1999). Both type I and type II PKA are expressed in the mammalian brain (Cadd and McKnight, 1989). In the absence of cAMP, PKA consists of an inactive heterotetramer of two catalytic subunits bound to two regulatory subunits (Taylor et al, 1990). Each regulatory subunit contains two tandem cAMP binding sites, a high affinity

site and a low affinity site (Taylor et al., 1990). Activation of PKA occurs upon increasing cAMP levels. Accordingly, cAMP binding to these two sites located on the R subunits, results in the dissociation of the holoenzyme and the release of the monomeric C subunits which in turn can phosphorylate serine and threonine residues on targeted proteins (Taylor et al., 1990, Wang et al., 1991, Gibbs et al., 1992). The C subunits are broad-spectrum serine/threonine kinases that could potentially target numerous proteins, therefore signalling specificity for PKA is required. A basic specificity of PKA action is supported by the fact that there is a specific subcellular localization of PKA isoforms. For example, RI is found throughout the cytoplasm, whereas RII is localized to nuclei, nucleoli, the microtubule organizing center, the Golgi apparatus, and the plasma membrane (Griffiths et al., 1990, Li et al., 1988). Interestingly, even within the same subcellular compartment the PKA isoforms can have a differential localisation. For example RIIα and RIIβ have been demonstrated to localize differently in the Golgi-centrosomal area (Keryer et al., 1999). The specificity of PKA action can also be achieved by its affinity to cAMP. The PKA isoforms become activated at different concentrations of cAMP: type Iα is activated when the cAMP concentration reaches 10 nM, type Iβ at 100 nM, type IIα at 200 nM and type IIβ at 600 nM (Taylor et al, 1990). However, a more fine-tuned specificity for PKA action is provided by the family of A-kinase anchoring proteins (AKAP) which specifically tether PKA via the R subunits to unique subcellular sites within the cell. It is now well established that PKA regulates many vital processes through its reversible phosphorylation of proteins. These processes include cellular metabolism, gene expression, cell and tissue development, morphogenesis, ion channel conductivity, synaptic transmission, and cell motility (Sutherland, 1972, Scott, 119, Taylor et al., 1990).

3. PKA in learning and memory

Research into the signal transduction cascades involved in learning and memory has given us a more detailed understanding of how these processes take place in the brain and it became clear that protein kinases play a crucial role. Among these kinases, PKA emerged as an important molecule in both synaptic and behavioral changes necessary for learning

and memory. Pioneering studies conducted in Kandel's laboratory showed for the first time that stimulation of PKA was necessary for the consolidation of long term memory in Aplysia (Schacher et al., 1988). The involvement of PKA in learning and memory was demonstrated later on also in Drosophila. In a molecular-genetic approach, inducible transgene expression of a gene encoding a peptide inhibitor of PKA (an N-terminal regulatory subunit fragment containing a pseudosubstrate inhibitory domain, and a wild-type catalytic subunit) impaired associative learning in transgenic flies (Drain et al., 1991). Subsequently and not surprisingly, a large number of studies reported the involvement of PKA in learning and memory in various species. For example, Zhao and colleagues showed that inhibitors of PKA impair long-term memory formation in chicks (Zhao et al., 1995). Moreover, studies by Romano and colleagues showed that PKA plays a key role in long-term memory storage in long term habituation (a gradual decrease in the response to a repeated irrelevant stimulus) in the Chasmagnathus crab (Romano et al., 1996). Overall, these experiments have laid much of the conceptual foundations for the subsequent research on the role of PKA in learning and memory in the mammalian brain. The most compelling evidence for the role of PKA in learning and memory in rodents came from the lab of T. Abel. Using a genetic approach, Abel and colleagues generated transgenic mice that express a dominant negative form of the regulatory subunit of PKA in the postnatal excitatory neurons within the forebrain (Abel et al., 1997). These mice exhibited normal initial learning in the hidden platform version of the water maze, where mice learn to locate a submerged platform during repeated trials, but showed deficits in memory during the retrieval test (Abel et al., 1997). While these experiments clearly showed the involvement of PKA in learning, the repeated training trials do not allow a discrimination between short-term and long-term memory. However, behavioral tests such as contextual fear conditioning, a single trial learning task in which animals learn to associate fear with a particular neutral context (e.g. conditioning box), allow to discriminate between short and long-term memory. In fear conditioning, the regulatory subunit PKA dominant negative transgenic mice showed deficits in long term memory, whereas short-term memory was not affected (Abel et al., 1997, Bourtchouladze et al., 1998). Studies on both amnesic patients and experimental animals showed that the hippocampus is a brain structure instrumental in the formation of memories. By using the tetracycline inducible system, Isiegas and

colleagues showed that genetic inhibition of PKA in the hippocampus during adulthood selectively impairs contextual fear long-term memory (Isiegas et al., 2006). In addition, several pharmacological studies also provided evidence for a role of PKA in learning and memory. For example, intra-ventricular or intra-hippocampal injection of PKA inhibitors were reported to impair long term contextual memory (Bourtchouladze et al., 1998, Wallenstein et al., 2002, Ahi et al., 2004). Altogether, these data show a fundamental role for PKA in learning and memory.

4. Compartmentalization of PKA signaling through AKAPs

It recently emerged that multifunctional binding proteins oversee the dynamic organization of the when and where signaling events take place, by clustering activator proteins with enzymes such as kinases, phosphatases and phosphodiesterases and directing them towards their downstream effectors. Particularly AKAPs play a crucial role in the dynamic organization of cellular events. To date, more than 50 AKAPs have been identified in a wide range of species, tissues and cellular compartments (Fig. 1) (Angelo and Rubin, 2000, Jackson and Berg, 2002, Sarkar et al., 1984, Wong and Scott, 2004). All members of the AKAP family share three common characteristics. First of all, all AKAPs contain an amphipathic helix of 14-18 residues, which binds to the N-terminal dimerization and docking domain of the regulatory subunits of PKA (Carr et al., 1991; Herberg et al, 2000; Newlon et al., 2001). Second, AKAPs have a unique subcellular targeting domain which directs the PKA-AKAP complex to defined subcellular structures and finally, each AKAP can interact with several other signaling proteins such as protein phosphatases or various signal termination enzymes. The AKAP family can be divided in three groups on the basis of which PKA regulatory subunits they are able to bind. Although the majority of AKAPs binds the RII subunits of PKA, RI AKAPs or dual AKAP that bind both RI and RII were characterized. Interestingly, it is believed that up to 70% of the intracellular PKA is bound to AKAPs.

AKAPs have e.g. been shown to participate in macromolecular signaling complexes that include protein kinases (serine/threonine and tyrosine kinases), phosphatases,

phosphodiesterases (PDE), adenylyl cyclases, adaptor molecules or ion channels, and also at least one member of the superfamily of G-protein-coupled receptors (GPCR) (Wong and Scott, 2004). Moreover, recent data show a role of PKA anchoring in several physiological processes including glutamate receptor trafficking (Westphal et al., 1999), synaptic function (Rosenmund et al., 1994), hormone-mediated insulin secretion (Lester et al., 1997), learning (Moita et al., 2002), cardiomyocyte contractility (Fink et al, 2001) and vasopressin-mediated translocation of aquaporin-2 into cell membranes of renal principal cells (Klussmann et al., 1999).

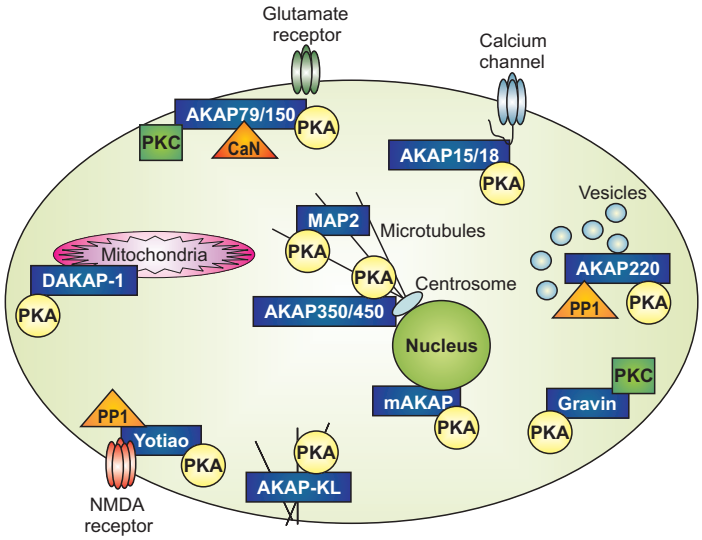


Fig. 1 AKAPs in different subcellular compartments. All AKAPs share the same characteristics: they all bind PKA, have a unique subcellular targeting domain and form a signalosome (adapted from Edwards and Scott, Curr Opin Cell Biol, 2000)

It is generally believed that spatio-temporal configurations of distributed cAMP/PKA activity in the brain are a major factor in changes in the strength of synaptic contacts between nerve cells, also known as synaptic plasticity. Synaptic plasticity is widely considered to be the cellular mechanism that underlies cognitive processes. It emerges from changes in neuronal transmission, both in the short-term and the long-term. Short-term synaptic plasticity depends only on covalent modifications of pre-existing proteins (mainly

protein phosphorylation/dephosphorylation by protein kinases and phosphatases) whereas long-term synaptic changes require new protein synthesis and are associated with the growth of new synaptic connections. Although much work has been put in revealing the identity of the molecules involved in synaptic plasticity, we are only starting to discover the mechanisms by which the multifold of intracellular signals during cognitive processes are regulated and coordinated by AKAPs.

In this thesis we focus on the role of two AKAPs in the brain: neuronal A-kinase anchoring protein 79/150 (AKAP79/150) and mAKAP. Whereas coordination of cAMP signaling by AKAP79/150 is mainly related to short-term synaptic plasticity (e.g. the dynamic protein phosphorylation of the AMPA receptor (Dell'Acqua et al., 2006), we have good reasons to assume that the recently identified mAKAP is a major player in processes that affect long-term synaptic plasticity such as the induction of gene expression. Both AKAPs are likely to play a role in coordinating synaptic plasticity and learning and memory considering their location and/or their signaling constituents.

5. A-kinase anchoring protein 150

One of the most prominently investigated AKAPs in the brain is AKAP79/150. This family of proteins consists of three orthologues: bovine AKAP75, murine AKAP150 and human AKAP79. Initially AKAP75 was identified as a contaminant of PKA RII purified from cytosolic brain preparations (Bregman et al., 1991; Sarkar et al., 1984). In addition, Bregman and colleagues obtained AKAP150 by screening a rat cDNA library using radiolabeled RIIβ as functional probe (Bregman et al., 1989). Finally AKAP79 was identified as a constituent of postsynaptic densities (PSD) in human cerebral cortex (Carr et al., 1992). All three proteins are highly related and differ only in their molecular weights, which is predominantly a consequence of repeat sequences present in the larger AKAP150. This prototypic anchoring protein consists of a positively charged N terminal region which is essential for its intracellular targeting, and of a high-affinity binding site for RIIβ near the C terminus. In addition to PKA-RIIβ, binding sites for protein kinase C (PKC) and protein phosphatase 2B (PP2B, calcineurin) were mapped. As a consequence, AKAP79/150

complexes can respond to intracellular second messengers such as cAMP, calcium and phospholipids (Klauck et al., 1996) influencing various signaling events.

It was showed that AKAP150 is abundantly expressed in the rat brain and is specifically enriched in postsynaptic densities (PSD) of excitatory glutamatergic synapses (Kennedy et al., 1997, Ziff et al., 1997, Yamauchi et al., 2002, Malenka et al., 2004). Here, AKAP79/150 targets its anchored proteins via postsynaptic density (PSD)-95 family membrane-associated guanylate kinase (MAGUK) scaffold proteins and forms a multi protein complex with AMPA and NMDA receptors (AMPAR and NMDAR) (Colledge et al., 2000), synaptic adhesion molecules, and cytoskeleton proteins that may play important roles in regulating synaptic structure and receptor function in synaptic plasticity. Although a few studies have been able to ascribe the regulation of specific neuronal events to a particular AKAP, electrophysiological studies established a role for AKAP79/150 in the modulation of ion channels such as AMPAR, L-type calcium channels and various potassium channels (Tavalin et al., 2002, Hoshi et al., 2003, Oliveria et al., 2007). In summary, the AKAP79/150 family of proteins emerged as key coordinators of various signaling events at postsynaptic densities in neurons which may affect both synaptic plasticity and learning and memory.

6. Muscle AKAP

Muscle AKAP (mAKAP), also known as AKAP100 or AKAP6, was first identified by expression cloning of a truncated cDNA that encoded a 100-kDa mAKAP fragment (McCartney et al., 1995). After further characterization of the protein, it was found that mAKAP was much larger than originally thought (Kapiloff et al., 1999; McCartney et al., 1995). Due to alternative splicing, two forms of mAKAP exist: mAKAPα and mAKAPβ. Both mAKAP variants are identical except for a 244 amino acid residue N-terminal extension in mAKAPα (Michel et al., 2005). Another difference between the mAKAP proteins resides in its localization. The longer form, mAKAPα, is preferentially expressed in the brain, whereas mAKAPβ is abundant in cardiac myocytes and skeletal muscle (Michel et al., 2005). In the heart mAKAP is targeted to the nuclear envelope through the

binding of three spectrin repeat domains, where it forms a large macromolecular signaling complex containing several signal transduction molecules, including PKA (Kapiloff et al, 1999, Kapiloff et al, 2001), the phosphodiesterase PDE4D3 (Dodge et al., 2001), the protein phosphatases PP2A and calcineurin (Kapiloff et al, 2001, Pare et al., 2005), ryanodine receptors (RyR2) (Kapiloff et al, 2001, Marx et al., 2000, Ruehr et al., 2003), the small GTPase Rap1, the guanine exchange factor Epac1 (Dodge-Kafka et al., 2005), and the mitogen-activated protein kinases (MAPK) MEK5 and ERK5 (Dodge-Kafka et al., 2005). Both in brain and heart, mAKAP can also bind 3'-phosphoinositide-dependent kinase 1 (PDK1) and p90 ribosomal S6 kinase (RSK3) (Michel et al., 2005). While in the heart mAKAP regulates cardiac function by converging MAPK, calcium, and cAMP signaling, the role of mAKAP in the brain remains so far rather unexplored.

It is very likely that mAKAP in the brain has a similar function as in the heart namely the integration of cAMP, calcium and MAPK pathways to regulate neuronal processes. Besides its localization at the nuclear membrane, there are several lines of evidence indicating that mAKAP in the brain is involved in long-term synaptic plasticity and in this way affects cognition. Long-term changes in synaptic strength are mediated through alterations in gene expression via the activation of transcription factors such as NFAT and cAMP responsive element binding protein (CREB). Like in the heart, calcineurin, which is anchored to mAKAP, mediates NFAT translocation to the nucleus and this is critically dependent on increased intracellular calcium levels in neurons (Graeff et al., 1999). Furthermore, several effectors of the mAKAP complex including intracellular calcium release and the ERK pathway are known to affect PKA-induced CREB phosphorylation and CREB-dependent transcription (Zanassi et al., 2001). Thus it can be expected that mAKAP is crucial for coordinating gene expression at the nuclear membrane and in this way plays an important role in numerous brain functions such as learning and memory.

7. Role of AKAPs in learning and memory

Although extensive research has already been performed on the role of PKA in the molecular mechanisms of learning and memory, limited data exists on the role of anchoring

proteins or anchored PKA. In addition to PKA, AKAPs provide platforms for the assembly of several other signaling enzymes known to be involved in learning and memory (e.g. PP2B, PP1 and PKC). As a consequence, this assembly of highly dynamic signaling enzymes within ionotropic and metabotropic glutamate receptors (AMPAR, NMDAR) and different subcellular compartments including synaptic vesicles, PSD and the cytoskeleton (reviewed by Wong and Scott, 2004) makes it likely that AKAPs are involved in learning and memory. In the brain, the majority of AKAPs tether the RII subunit of PKA. In the mammalian central nervous system, RIIβ (highly expressed in the amygdala and hippocampus) is the predominant isoform and principal mediator of cAMP action (Sarkar et al., 1984). Interestingly, mice with a targeted disruption of the RIIβ gene of PKA in the amygdala showed impaired long-term memory in a conditioned taste-aversion test. The conditioned taste-aversion test is an amygdala dependent classical conditioning test, in which lab animals learn to associate a novel taste of certain food with symptoms caused by a toxic, spoiled, or poisonous substance (Koh et al., 2003).

Initial evidence for a role of AKAPs and PKA anchoring in learning and memory came from a study by Moita and colleagues, who blocked PKA anchoring in the lateral amygdala of rats and subjected the animals to auditory fear conditioning (Moita et al., 2002). Auditory fear conditioning is a classical Pavlovian associative emotional learning task in which mice are presented with an auditory cue (conditioned stimulus (CS)) followed by a short mild electric shock (unconditioned stimulus (US)). In the subsequent retention, if mice learn the task, they will exhibit a clear conditioned fear response, i.e. freezing behavior (defined as the lack of movement except for respiration and heart beat) to the auditory cue (CS). In Moita's study, rats received bilateral infusions of St-Ht31 (a peptide that blocks PKA anchoring) or vehicle solution in the amygdala, 1 hour before auditory fear conditioning. The memory for the tone was assessed 1, 4 and 24 hours after training by measuring freezing behavior. Rats showed intact freezing 1 hour after training but impaired freezing both 4 and 24 hours after training, suggesting a role of PKA anchoring in the consolidation, but not the acquisition of auditory fear memory (Moita et al., 2002). More evidence for the role of anchored PKA in learning was provided by Schwaerzel and colleagues in Drosophila. They showed that, AKAP-bound PKA is required for aversive memory in a Pavlovian olfactory learning task, in which an electric shock is used as an

aversive unconditioned stimulus (Schwaerzel et al., 2007). Moreover, it has been shown that AKAPs play an important role in neuronal synaptic plasticity (Bauman et al, 2004). However, many questions still remained unanswered. For example, what is exactly the contribution of AKAPs and the AKAP signalosome to short-term and long-term memory? What is their contribution in the different stages of learning and memory (e.g. acquisition, consolidation, retrieval or extinction)? Are AKAPs involved in different memory systems (e.g. declarative memory, non-declarative memory)? Which AKAP is involved in learning and memory? Progress in the field of AKAPs will lead to integrated knowledge on AKAP scaffolding in cognitive processes under physiological and pathological conditions. Ultimately this knowledge on the coordination of signaling cascades may lead to the development of novel, more fine-tuned, innovative therapeutic strategies to treat cognitive dysfunction.

8. Tools for AKAP research

There are several ways to investigate the function of AKAPs and the tethering of signaling enzymes to AKAPs. It can be done by peptides that block the binding of enzymes to the complex or via genetic modifications which imply either to knock down of a particular AKAP or to generate truncated forms of a particular AKAP which lack the ability to bind particular signaling enzymes.

To determine whether PKA anchoring to AKAPs has profound implications on PKA function, several peptides which are able to compete for PKA anchoring to AKAPs, were engineered. Carr and colleagues developed the first synthetic peptide covering amino acid residues 493 to 515 of the thyroid AKAP Ht31 (Carr et al., 1992). Ht31 is able to compete for both RIIα (Carr et al., 1992) and RIIβ as well as for RIα anchoring (Herberg et al., 2000). As an inactive control for this peptide a peptide named Ht31-P was developed. Ht31-P has two isoleucine residues substituted by proline residues which disrupt the amphipathic helix structure necessary for R-subunit binding. Although the Ht31 peptide proved to be an excellent tool to ascertain the role of PKA anchoring in various cellular processes, it remained unclear which of the PKA isoforms as well which AKAP was

responsible. To overcome this limitation, PKA isoform specific anchoring competing peptides were developed. Using computer-based and peptide array screening approaches, Alto and colleagues generated a novel PKA binding competitor named AKAP-in silico, a high-affinity RII selective binding peptide (Alto et al., 2003). Studies aimed to develop even more potent and selective peptide anchoring competitors soon followed. Gold and colleagues developed superAKAP-IS, a peptide that is 10,000-fold more selective for the RII isoform relative to the RI subunit of PKA (Gold et al., 2006). Moreover, by using a combination of bioinformatics and peptide array screening Carlson and colleagues developed a high affinity-binding peptide called RIAD (RI anchoring disruptor) which is more than 1,000-fold more selective for type I PKA over type II PKA (Carlson et a., 2003). To date, there is a broad array of PKA anchoring disrupting peptides available (Hundsrucker et al., 2006) and numerous research groups showed that these peptides are valuable tools to study the relevance of PKA anchoring in various cellular processes. However, although PKA isoform specific anchoring disrupting peptides are available, these are not specific for a particular AKAP. The interaction of PKA RII subunits with various AKAPs is shown to involve an amphipathic helix motif of 14-18 residues with a conserved structure in the AKAP where the hydrophobic face binds the RII dimer (Carr et al., 1991; Vijayaraghavan et al., 1999). However, the number and distribution of hydrophobic amino acid residues in RII-binding domains is similar in all AKAPs (Hundsruker et al., 2006). Therefore, while numerous PKA anchoring peptides were designed, developing AKAP specific PKA anchoring disruptors remains very difficult (Hundsrucker et al., 2006). To be able to place a particular AKAP in a particular physiological response, AKAP specific PKA anchoring disruptors need to be developed. Unfortunately, except PKA anchoring disruptors, inhibitory anchoring peptides for other AKAP complex members such as e.g. protein phosphatases are still not available. AKAP site-specific mutants lacking the ability to anchor PKA and/or other signaling enzyme would lead to a better understanding of spatial and temporal organization of signaling events. In the lab of J. D. Scott, deletion mutants lacking enzyme-binding sites for protein kinases A (PKA), protein kinase C (PKC), or protein phosphatase 2B (PP2B) were already used to assess the role of AKAP79/150 signalosome in modulating neuronal ion channels activity (Hoshi and Scott,

2006). Overall, further investigation into the role of AKAPs and anchoring of signaling enzymes will reveal novel roles for accurate intracellular signaling in learning and memory.

9. Exchange protein directly activated by cAMP

A long time it was believed that the major effects of cAMP in mammalian cells were mediated by PKA or cyclic-nucleotide gated ion channels (CNGs). However, fairly recently, a novel family of cAMP sensor proteins named exchange protein directly activated by cAMP (Epac) or cAMP-regulated guanine exchange factor (cAMP-GEF) was characterized (de Rooij et al, 1998, Kawasaki et al., 1998). This family consists of two multi-domain isoforms, named Epac1 (cAMP-GEF-I) and Epac2 (cAMP-GEF-II) which are products of independent genes in mammals. These proteins share extensive sequence homology and both contain an cAMP binding domain (CBD) that is homologous to that of PKA R subunits. It consists of an N-terminal regulatory region and a C-terminal catalytic region. While Epac1 has one CBD, Epac2 possesses a similar additional domain with a so far unknown biological function (Bos, 2006). Another difference between the two Epac variants arises from the expression of the proteins in various tissues. It has been shown that Epac1 mRNA is ubiquitously expressed in all tissues while Epac2 mRNA is predominantly expressed in adrenal glands and brain tissue (Kawasaki et al., 1998). In addition, fluorescent microscopy studies revealed that Epac is present in the nuclear membrane, plasma membrane and mitochondria and subcellular redistribution of Epac appears to be regulated during the cellular cycle (Qiao et al. 2002).

By serving as an cAMP-binding protein, Epac1 and Epac2 couple cAMP production to the activation of Rap1 and Rap2, small molecular weight GTPases of the Ras family. To date, several studies demonstrated the role of Epac in various cellular processes such as cell adhesion (Enserink et al., 2004, Kooistra et al, 2005), exocytosis (Ozaki et al., 2000), secretion (Kiermayer et al., 2005), cell differentiation (Bryn et al., 2006; Shi et al. 2006), cell proliferation (Misra and Pizzo 2005), apoptosis (Kwon et al., 2004), gene expression (Sands et al., 2006, Ulucan et al., 2007) and cardiac hypertrophy (reviewed by Roscioni et al., 2008). Despite of the growing body of literature, the role of Epac in the brain remained

largely unknown. It is surprising that in view of the role of cAMP in brain functions (e.g.: axon growth and regeneration, addiction, synaptic, plasticity, and memory), few data are available on the role of Epac in the central nervous system.

Epac was shown to enhance neurotransmitter release in glutamatergic synapses (Sakaba and Neher, 2003; Zhong and Zucker, 2005; Gekel and Neher, 2008), whereas in cultured cerebellar granule cells it can modulate neuronal excitability via Rap and p38 MAPK activation (Ster et al., 2007). Moreover, in dorsal root ganglion Epac mediates the translocation and activation of protein kinase C (PKC) leading to the establishment of inflammatory pain (Hucho et al., 2005).

Thus far, evidence for a role of Epac in the process of learning and memory is limited. However, since Epac is a cAMP-responsive enzyme and cAMP signaling is established to be of critical importance in learning and memory, an involvement of PKA-independent cAMP signaling through Epac proteins can be expected. The first indications for a role of Epac in hippocampus-dependent learning and memory came from very recent studies. Gelinas and colleagues reported that Epac activation enhances the maintenance of LTP in area CA1 of mouse hippocampal slices (Gelinas et al., 2008) and co-application of a selective PKA and a selective Epac activator was shown to rescue the memory retrieval impairment observed in dopamine-beta-hydroxylase deficient mice (Ouyang et al., 2008).

These initial findings indicate a likely and important role for Epac in learning and memory processes. However, the contribution of Epac1 and Epac2 in the molecular machinery of learning and memory remains to be elucidated. As such, the involvement of this additional highly coordinated cAMP effector in learning and memory represent a major research interest for future cAMP-mediated signaling studies.

10. Aim and outline of the thesis

Memory is an amazing feature of the (human) brain. It is not only defining who we are but it will also dictate who and what we are going to become. Memory has always fascinated human kind. E.g. before Christ the Greek goddess of memory was called Mnemosyne. Over time, a large number of philosophers studied the nature of memory. However, the modern

research on learning and memory started in 1887 with the work of Joseph Jacobs on short term memory (Jacobs, 1887). Although advances in the field of learning and memory have been made due to behavioral, electrophysiological, molecular, pharmacological and biochemical experiments, the quintessence of how the brain functions in learning and memory remains elusive. Memory and learning are so closely connected that people often confuse them with each other. However, learning and memory are not unitary processes. Learning is the process by which new information is acquired; memory is the process by which that knowledge is retained and remembered. For example: you learn a new language by studying it, but you then speak it by using your memory to retrieve the words that you have learned.

The major aim of this thesis is to conduct fundamental research in the field of learning and memory. We put a specific emphasis on the role of the second messenger cyclic AMP (3'-5'-cyclic adenosine monophosphate) in the molecular mechanisms underlying memory formation. Understanding this complex process does not only provide fundamental insights, but will also lead to the development of new therapeutic strategies for improving cognition in both neurodegenerative disease and in normal age-dependent cognitive decline.

The main downstream effector of cAMP is PKA (cyclic AMP-dependent protein kinase). PKA was found to be involved in stimulus-induced changes in synaptic strength, a process called synaptic plasticity which is believed to provide at least in part the cellular basis of learning and memory. Moreover, PKA was established to be a key enzyme in learning and memory processes. However, it remained unclear for a long time how such a broad enzyme can discriminate between different stimuli and respond accordingly. Fairly recently a key mechanism by which PKA achieves signaling specificity was reported. Specificity resides from subcellular targeting of PKA through scaffolding or adaptor proteins. Indeed, it is believed that up to 70% of the intracellular PKA is bound to a family of proteins called A-kinase anchoring proteins (AKAP). One of the AKAPs expressed in the rodent brain is AKAP150. In addition to PKA, AKAP150 was found to target protein kinase C (PKC) and protein phosphatase 2B (PP2B) to the neuronal membrane, particularly of synapses (Carr et al., 1992).

Although it is widely acknowledged that all the complex members of AKAP150 play a crucial role in synaptic plasticity and learning and memory, little is known about the role of AKAP150 itself in these processes. Therefore, our first aim was to investigate the role of AKAP150 in learning and memory. To get a first indication of the function of AKAP150 we investigated the distribution of AKAP150 in the mouse brain (**Chapter 2**). Interesting, AKAP150 mRNA was shown to be upregulated in the hippocampus, 3-12 hr after the induction of LTP (long term potentiation), a long-lasting enhancement in synaptic efficacy (Genin et al., 2003). This hippocampus is a brain structure known to be critically involved in memory formation. In **chapter 3** we raised the question whether AKAP150 protein levels in the mouse hippocampus, change during learning. For this, we used a learning paradigm, named contextual fear conditioning, in which mice learn to fear the context (conditioning box) in which the experiment is performed. This is done by pairing the conditioning box with an aversive mild electric foot shock (Fig. 2A). Eventually, the conditioning box alone can elicit the state of fear. Conditioned fear reflects emotional associative memory in mice and is measured as freezing, immobility-like behavior, except the movement created by breathing.

Besides determining whether the levels of AKAP150 change in learning and memory, it is at least as interesting to explore whether the anchoring of PKA to AKAP is important in learning and memory. Therefore, in **chapter 4** we investigated the importance of PKA anchoring to AKAPs in the different stages of contextual fear conditioning: acquisition (encoding of memory), consolidation (storing and maintaining of memory), retrieval (accessing of memory) and extinction ("erasing" of memory). To address this question, we employed a pharmacological approach. Mice were injected intracerebroventricularly or intrahippocampally with membrane permeable PKA anchoring disrupting peptides at different time points during the memory process (Fig. 2B).

To date, the AKAP family of proteins consists of more than 50 members and several of these AKAPs have been found in the brain. Initially, muscleAKAP (mAKAP) was reported to be present in the heart and skeletal muscles where it coordinates cAMP, calcium and MAPK (mitogen-activated protein kinase) signaling (Dodge-Kafka and Kapiloff, 2006). However, mRNA studies showed that a splice variant of mAKAP is also expressed in the

brain. Because mAKAP signaling complex members in the heart are involved in numerous brain functions, it is very likely that in the brain, mAKAP also integrates several signaling pathways to regulate neuronal processes. In **chapter 5** we aimed to answer the following questions: What is the brain distribution of mAKAP? Does mAKAP expression changes during ageing? What may be the function of mAKAP in the brain?

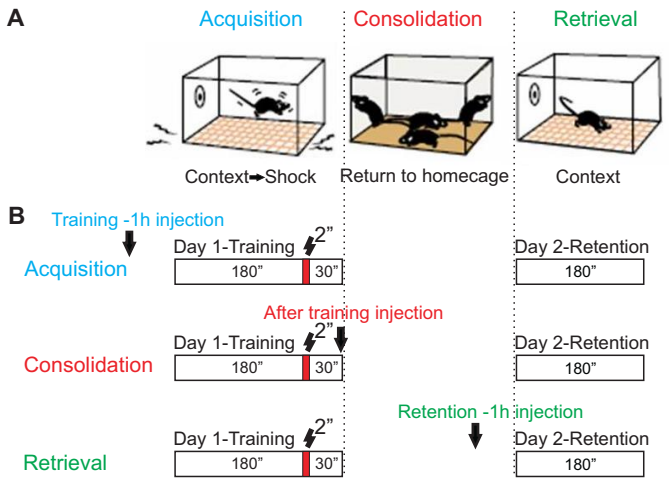


Fig. 2 Contextual fear conditioning experimental setup. A. Different stages of fear memory can be distinguished: acquisition, consolidation and retrieval. B. Acquisition, consolidation and retrieval can be affected by injecting pharmacological compounds in the mouse brain at different time points during fear conditioning (adapted from Abel and Lattal, Curr Opin Neurobiol, 2001).

Although PKA was long thought to be the main effector of cAMP, fairly recently another cAMP effector was identified. cAMP can also activate Rap-guanine nucleotide exchange factor protein directly activated by cAMP (Epac). Because cAMP signaling is crucial in learning and memory, an involvement of PKA-independent cAMP signaling through Epac proteins can be expected. In **chapter 6** of this thesis, we explored the role of Epac in learning and memory by pharmacological activation or gene silencing of Epac in the mouse brain in several learning paradigms. Accordingly, a cAMP analogue which specifically activates Epac but not PKA was delivered into the mouse brain and its role in the acquisition, consolidation, retrieval and extinction of contextual fear conditioning was

determined. The data found in contextual fear conditioning were confirmed in passive avoidance, another fear motivated learning task. To exclude effects of Epac activation on anxiety we also investigated the effect of Epac activation on axiety behavior in the elevated plus maze. From the distribution of the two Epac isoforms, Epac1 and Epac2, we deduced that Epac2 is abundant in the mouse brain whereas Epac1 is hardly present. Therefore, we studied the role of reduced Epac2 expression in immediate and delayed fear memory.

Chapter 7 summarizes and discusses all findings of this thesis and provides an overall conclusion and future perspectives.

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